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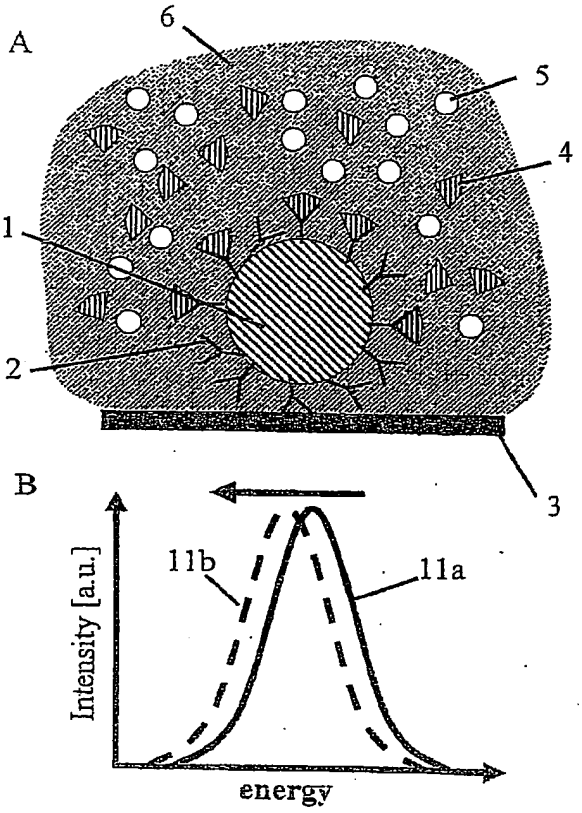
(54) Title: METHODS, DEVICE AND INSTRUMENT FOR DETECTION OF ANALYTES



(57) Abstract: The present invention relates to a method, a device and an instrument for the detection of an analyte (4) in a sample(6). Such method, device and instrument is used in clinical diagnostics, pharmacology, e.g. drug screening, environmental analysis as well as in other fields of analysis like chemistry or biochemistry. According to the present invention, the method for detection of an analyte (4) in a sample comprises the following steps of a) providing at least one particle structure (1) detectable by light scattering and coated with binding entities (2) for said analyte (4); b) contacting the at least one particle structure (1) with the sample (6), c) illuminating the at least one particle structure (1) and measuring the scattered light emitted by individual particle structures (1) at least twice and d) determining a change in the spectral signature of the scattered light as a measure of the presence and/or quantity of the analyte (4) in the sample (6). In order to detect more than one analyte (4) simultaneously particle structures (1) of different classes of particle structures maybe coated with different binding entities. Further, instead of coatig the particle structure (1) with binding entities specific for the analyte (4), the particle structure (1) may be coated with substances (21) competing with said analyte (4) for binding to specific binding entities (2). Any competitive and non-competitive assay format can be put into practice.

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METHODS, DEVICE AND INSTRUMENT FOR DETECTION OF ANALYTES

Description of **WO2004042403**

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Methods, Device and Instrument for Detection of Analytes The present invention relates to a method, a device and an instrument for the detection of analytes in a sample. Such method and apparatus is used in clinical diagnostics, pharmacology, e. g. drug screening, envi- ronmental analysis as well as in other fields of analysis like chemistry or biochemistry. The method of the present invention is particularly useful for detection of haptens, peptides, proteins, antigens, polynucleotides, antibodies or fragments thereof, oligonucleotides like DNA or RNA.

Molecular recognition has led to a revolution in clinical diagnosis as well as in pharmacology during the last decades.

It is known from the prior art to measure particle plasmon resonance for detection of analytes in a test solution. Such prior art is described in US 6,214, 560, in US 6,180, 415, as well as in Englebienne, P. The Analyst 123,1599-1603 (1998) and in Haes and van Duyne, J Am. Chem. Soc. 124,10596-10604 (2002).

US 6,214, 560 discloses a binding assay for detection of an analyte using scattered-light detectable particles with a size between 1 and 500 nm. This assay comprises binding a scattered-light detectable particle to analyte molecules and illuminating the parti- cle with a light beam. The light scattered by the particle is then observed as a measure of the pres- ence of the analyte as the particle appears as a bright object on a dark background. Coating of the

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particle by monolayers of binding agents or other material did not noticeable alter the light scattering properties specific for that type of particle. Thus, the scatterd-light detectable particle is used as a marker of analyte molecules.

US 6,180, 415 discloses a further method and apparatus for detecting the presence and/or amount of an analyte in a sample. As a diagnostic method the sample is preferably contacted with plasmon resonance entities (PREs) to produce a ligand/ligand-binding complex between the analyte molecules and the PRE. This target is then illuminated and a spectral characteristic of the emitted scattered light is observed.

PREs with a selected spectral signature are discriminated from other light-scattering entities based on detected spectral characteristic values unique to the selected PREs. The occurrence and quantity of the PRE-analyte-complexes on a substrate can be determined and counted.

Thus, spectral differences in plasmon resonance characteristics between different PREs on the same substrate are evaluated as analytical tool. Neither US 6,214, 560 norUS-6, 180,415 disclose or suggest comparative measurements of the spectral characteristics of a particle before, during and/or after an analyte binding event.

Englebienne reports the measurement of surface plasmon resonance of colloidal gold particles with a diameter of about 40nm. These gold particles were coated with various antibodies. Upon addition of corresponding ligands a shift in surface plasmon resonance of the gold particles of up to 3nm was detected. As all measurements have been done using a

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commercial clinical chemistry automated analyzer, Englebienne records surface plasmon resonance from a colloidal emsemble of gold particles but not from a single particle. Similar ensemble measurements with increased sensitivity have also been reported by Haes and van Duyne,J Am. Chem. Soc. 124,10596-10604 (2002).

However, as the plasmon resonance frequency is strongly dependent on the size, shape or other parameters of the nanoparticles inhomogeneous broadening is observed when measuring a particle emsemble.

Such inhomogeneous broadening makes it difficult to detect small shifts of plasmon resonance caused by binding of ligands to a particle. Englebienne does not observe changes of the spectral characteristics of a single nanoparticle before, during and/or after a binding event.

Throughout this text the following terms are used: The term"particle structure"as recited herein refers to any independent structure or entity, exhibiting light scattering, e. g. a single particle, combination, association or arrangement of two or more particles exhibiting an individual spectral signature common to the single particle, combination, association or arrangement of particles.

As example, a single metal particle shows a unique individual particle plasmon resonance. Such a unique particle plasmon resonance is also observed if two or more resonant particles are arranged in close vicinity, e. g. with a distance between each other less than their diameter. In the latter case the particles of the ensemble interact with each other and exhibit

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common spectral signatures.

The term"spectral signature"refers to any characteristics of the emitted scattered light, e. g. spectral position of the maximum emission or intensity, bandwidth and so on, any combination of such characteristics or any result of any mathematical operation on such characteristics or combinations.

The term"binding entity"designates any partner of a specific binding pair, comprising for example antigen/antibody, hapten/antibody, ligand/receptor, carbohydrate/lectin, nucleic acid/complementary nucleic acid, nucleic acid/complementary peptide nucleic acid.

Nucleic acids comprise DNA, RNA, polynucleotides and oligonucleotides. Polynucleotides comprise more than 100 nucleotides, oligonucleotides comprise 5 to 99 nucleotides, preferably 5 to 50 nucleotides, preferably 6 to 30 nucleotides.

It is an aim of the present invention to provide a method, a device and an instrument for the rapid detection of one or more analytes in a sample with improved sensitivity and selectivity.

This object is solved by the method according to one of claims 1 to 4, the device according to claim 33 and the measuring instrument according to claim 51.

Improvements of the method, the device and the instrument of the present invention are described in the respective dependent claims.

With the present invention it is demonstrated for the first time that the shift of the plasmon resonance

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spectrum of a single, individual nanoparticle structure carrying specific binding entities can be detected in response to a binding event, thereby allowing the measurement of the presence and/or quantity of an analyte in a sample.

The method of the present invention is characterized by providing at least one scattered light detectable particle structure, wherein binding entities specifically binding the analyte are coated onto the surface of the particle structure. For measurement a single particle structure is illuminated with interrogating light and the scattered light emitted by this single individual particle structure is detected. For detection of the binding of an analyte molecule to the binding entity immobilized on the surface of this single particle structure the change in the spectral signature, e. g. a wavelength shift of the emission spectrum of the scattered light, caused by this binding event is detected and used as a measure for the binding event between analyte molecule and binding entity. On the other hand, displacement of a species bound to the surface of the particle structure by competitive binding of analyte molecules to the binding entities may also be detected, thereby detecting the presence or concentration of the analyte. Thus, a change in the spectral signature of the nanoparticle structures, e. g. a wavelength shift of the emission spectrum of scattered light is a measure of the presence of the analyte in the sample. The sample may be any fluid e. g. a test solution, liquid, or gas. Even measurement on the surface or within biological cells, e. g. in cell culture, are possible, e. g. for expression analysis and the like.

The particles may be deposited on a substrate. As a

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substrate any support may be used, e. g. solid supports like glass, resins, plastics, metal, films and/or gels.

As spectral signature particle plasmon resonance emission can be used. A particle plasmon resonance is a collective oscillation of the conduction band electrons in particles with dimensions of or less than the wavelength of the corresponding light frequency.

The particle structures according to the present invention may contain metals or semiconductors and have diameters between 1 and 500 nm (nanoparticles), preferably between 10 and 150 nm. If for example particle structures or particles with a diameter of 40 nm coated with antibodies are used, the number of binding entities immobilized on the surface of the particle structures is between 40 and 100. With such a small number of binding entities a very low concentration of the analyte in the sample is still detectable, if specific binding entities are immobilized on the surface of the particle structure as claimed in the present invention.

The binding entities may be any kind of reagents capable of specific molecular recognition interaction, such as antibodies, antigen binding fragments of antibodies, peptides or proteins, antigens, polynucleotides, oligonucleotides, peptide nucleic acids or artificial binding agents, which are specific for a certain binding substance (analyte). Thus, any kind of analyte, like antibodies, peptides, proteins, antigens, nucleic acids (oligonucleotides, polynucleotides, DNA, RNA), haptens or environmental pollutants may be detected.

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Binding events of analyte molecules to the binding entities may then change the dielectric properties, e. g. the refractive index or the surface charges, of the closest vicinity of the particle structures. This in turn leads to a change of the scattering spectrum of the particle structure.

The sensitivity of the method according to the present invention can even be improved if the sample is brought into contact with only a few nanoparticle structures coated with binding entities and the experimental conditions are such that the analyte molecules are harvested by the nanoparticle structures and accumulated on their surface.

As single nanoparticle structures are observed, different nanoparticle structures may even be separated by their spectral difference as for example due to different size, different structural characteristics, different material or different particle shape. Such separation provides for the possibility to conduct multiplex measurements for different analytes in the same sample if different classes of particle structures are coupled to different specific binding entities. Classes of particle structures may differ in particle size; shape, structural characteristics, material or composition.

The present invention can further be improved by measuring a plurality of single particle structures and afterwards averaging over all single measurements. Different to the prior art the averaging is done after multiple individual measurements of single particle structures instead of averaging over an ensemble of particles during the measurement as in the prior art.

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The signal detected from the single particle structure can further be improved by contacting the bound analytes with a further signal enhancing agent. Such signal enhancing agent may comprise a metal, alloy, semiconductor, glass, latex particle or organic substance, e. g. proteins, polysaccharides, nucleic acids, organic synthetic or natural polymers as label.

The labeling, e. g. gold labeling, further changes the nanoenvironment of the scattered light detectable particle and increases the wavelength shift of the emitted scattered light (sandwich arrangement).

The scattered light can be separated from the interrogating light (excitation light) by using dark field microscopy, reflection microscopy, total internal reflection microscopy or scanning near field optical microscopy (SNOM). By using a dark field microscope it is possible to observe a single nanoparticle and separate the light scattered from this particle from scattered light originating from other particles for measurement. It is then preferable to deposit particles on the substrate with a distance from each other of

at least the spacial resolution of an optical detection means.

The spectral signature of the scattered light may then be analyzed and recorded by a CCD camera, one or more photodiodes, photomultipliers and/or photodiode arrays in combination with a spectrometer, a monochromator and/or dichroic mirror.

It is one feature of the present invention that this detection method does not require the binding reaction to reach thermodynamic equilibrium, but measurements can also be taken in the kinetic phase of the

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binding reaction. This allows short assay times, i. e. in the range of seconds to several minutes.

In one embodiment of the present invention, a detector sensitive for one or several particular wavelengths or wavelength regions is used. If there occurs a shift in emission wavelength of the scattered light the intensity of the scattered light emitted at these certain wavelengths will change and such change can be recorded as quantity correlated to the presence of the analyte in the sample.

A method to determine changes in the spectral signature of the nanoparticle structure may be illuminating the particle structure at two different wavelengths, one redshifted and one blueshifted (i. e. at lower and at higher energy) to the resonance peak wavelength of plasmon scattering. By calculation of the ratio between the intensities at both wavelengths a measure for the spectral signature of the nanoparticle is obtained.

The scattered light detectable particle structures may consist or comprise metal or metal embedded in a surrounding dielectric material (shell). The metal of the particle structures shows a particle plasmon resonance, the strength and the relative position on the frequency or wavelength scale being dependent on various parameters like the radius of the metal core and the thickness of the first inner shell. However, it is also possible to use particle structures, wherein the inner core consists of or comprises a dielectric material and the shell consists of or comprises a metal with a particle plasmon resonance. The particle structures may therefore either contain or comprise a metal, a heavily doped semiconductor or a

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superconductor or any combination of these materials.

As metal platinum, palladium, gold, silver, copper, zinc, yttrium, vanadium, manganese, cadmium, selenium, lanthanum, cerium, samarium, europium, terbium or alkali metals may be preferably used.

By modifying the size or the shape of the particle structures plasmon resonance frequencies in the ultraviolet, visible or infrared spectral region may be generated. The particle structures preferably have a rod-like, sphere-like or ellipsoidal shape.

In the following section examples for embodiments according to the present invention are described.

Therein Fig. 1 describes the generation of a particle plasmon scattering wave; fig. 2 describes the origin of the wavelength shift due to binding events; fig. 3 describes the detection of nucleotides; fig. 4 describes the dependency between particle plasmon resonance characteristics and shell thickness; fig. 5 describes the detection of proteins by dark field microscopy; fig. 6 describes the use of different illumination technologies ; fig. 7 shows the use of a flow cell for detection of low concentrations of analytes;

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fig. 8 shows a schematic drawing of a flow cell according to the present invention; fig. 9 shows a photograph of a flow cell according to the present invention; fig. 10 demonstrates two ways of multiplex detec- tion according to the present invention; fig. 11 describes signal enhancement; fig. 12 shows the scheme of a competitive binding assay; fig. 13 shows the scheme of a further competitive binding assay; fig. 14 shows signal distributions acquired by a wave guide (Fig. 14A) and a dark field mi- croscope (Fig. 14B); fig. 15 demonstrates the effect of particle shape on the present invention; fig. 16 describes the use of a particle structure consisting of two closely spaced particles. fig. 17 demonstrates the kinetic development of the plasmon resonance signal shift due to BSA- digoxigenin binding to gold particles functionalized with antibodiesM19-11 ;fig. Ab</RTI> demonstrates binding of streptavidin to single gold nanoparticles functionalized with biotin-BSA; and

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fig. 19 describes the kinetic development of the plasmon resonance signal shift due to bind- ing of streptavidin to single gold nanopar- ticles as well as stability against unspe- cific binding.

Fig. 1 shows the principle of particle plasmon reso- nance generation. Here and in the following figures same or similar reference signs are used for same or similar elements.

In Fig. 1A reference sign 1 is a metal particle with a size between a few and some 100 nm. This particle 1 is excited with interrogating light 10 which excites a collective oscillation of the conduction electrons (particle plasmon resonance) of particle 1. This col- lective oscillation of the conduction electrons emits a scattered wave with a typical spectrum in the ul- traviolet, visible or infrared region. This particle plasmon resonance is different from 2-dimensional surface plasmon resonance.

Fig. 1B shows a typical single particle plasmon reso- nance spectrum 11 of a Lorentzian shape, wherein the scattering efficiency is plotted against the photon energy. The peak wavelength of the single particle spectrum critically depends on the local surrounding 6.

Fig. 2 schematically shows in Fig. 2A a nanoparticle 1, e. g. of gold, immobilized on a substrate 3. On the surface of this particle 1 antibodies 2 are immobi- lized which carry specific binding entities for ana- lyte molecules 4. When a solution 6 containing ana- lyte molecules 4 and molecules 5 is contacted with

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the particle 1 analyte molecules 4 will bind to the antibodies 2, whereas molecules 5 will not bind to the particle 1 or antibodies 2. By binding to the antibodies 2 analyte molecules 4 change the dielectric properties of the shell of particle 1 and thereby shift the particle plasmon resonance spectrum lla as shown in Fig. 2B, to become spectrumllb.

The binding entities immobilized to the surface of particle 1 are not restricted to antibodies but may also be oligonucleotides, which hybridize to a specific target oligonucleotide, e. g. a specific sequence range of a gene, which may contain a single nucleotide polymorphism (SNP). Fig. 3A discloses such a particle 1 with immobilized oligonucleotides 2 which constitute a shell 7 around particle 1 with a refractive index of nl. The particle plasmon spectrum lla emitted by such particle 1 is shown in the insert figure of fig. 3A. In fig. 3B the same particle 1 is shown with target oligonucleotides 4 hybridized to the immobilized probe nucleotides 2. By hybridizing, the dielectric constant as well as the refractive index around particle 1 is changed from ni to n2 thereby shifting the plasmon resonance spectrum from curve lla to curve llb as shown in the insert figure of fig. 3B.

Fig. 4 discloses the calculated influence of shells 7 of different thickness on the shift of the resonance maximum of the particle plasmon resonance spectrum.

In this case a silver particle 1 of 60 nm diameter has been assumed to be coated by a shell 7 of varying thickness and a refractive index of 1.42. This coreshell structure is embedded in water 6. Depending on the thickness of the shell 7 the resonance peak shifts between 420 nm and 432 nm. This shift of the

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particle plasmon resonance is most pronounced by in- creasing the thickness of the shell 7 up to 20nm.

This figure therefore demonstrates that only the closest vicinity of about 20 nm around the particle 1 is relevant for the particle plasmon resonance shift.

Therefore, it is possible to sense a volume of less than 0.1 attoliter by observing an individual single particle.

Fig. 5 shows in its part figures 5a to 5e the princi- ple and schematic representation of a highly sensi- tive immunoassay based on light scattering from a single gold nanoparticle. Fig. 5a shows a single gold nanoparticle 1 which is functionalized with antibod- ies 2 against an antigen. Without the first shell of antibodies 2 the refractive index around particle 1 in the sample is about ni = 1.3 whereby the shell 7 constituted by the antibodies 2 has a refractive in- dex of n2 = 1.5. In the last part of Fig. 5a antigen molecules 4 are bound to the antibodies 2 thus enlarging the shell with a refractive index ofn2= 1.5 and thus shifting the particle plasmon resonance spectrum. Fig. 5b shows the calculated scattering in- tensities lla of gold particles which are embedded in water 6 (curve lla), with a 7 nm thick shell 7 of re- fractive index 1.5 (curvelie) and a particle 1 with a 15 nm thick shell 7 of refractive index 1.5 (curve llb). Fig. 5c is a gray scale photograph of a sample of functionalized gold nanoparticles in dark field illumination. An

experimental set-up for dark field microscopy with an objective 8, incident light 10 as well as a scattered light 11 is shown in Fig. 5d and 5e. By such an arrangement only scattered light 11 scattered from an individual functionalized gold par- ticle 1 is collected by the objective 8, spectrally resolved by e. g. a grating spectrometer and detected

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e. g. by a nitrogen cooled CCD camera. As seen in Fig.

5e, after adding a suitable antigen 4 to liquid 6 specific binding to antibodies 2 occurs. In consequence, shell 7 with high refractive index around particle 1 is enlarged and a red-shift of the spectral position of the nanoparticle plasmon resonance is observed.

Figs. 6A and 6B disclose in more detail the principle of dark field illumination. In Fig. 6A incident light rays 10 pass through a dark field aperture 13 to a condenser lens 9 which focuses this interrogating incident light 10 onto the sample placed on a sample holder 3. Between the sample holder 3 and the condenser lens 9 an immersion oil drop 12 is placed. The scattered light 11 is then collected by objective 8 and measured, whereas the direct light does not enter the objective 8. Fig. 6B shows a different technique for irradiation in a dark field microscope with direct incident light 10 impinging on the substrate 3 from above.

In Fig. 6C the illuminating light 10 passes through a beam splitter 25 and an objective 8 before illuminating the particle 1 on the substrate 3. The scattered light 11 passes again through the objective 8 and is then reflected by the beam splitter to a detector.

The scattered light 11 is thus separated from the incident light and carries the spectral signature of particle 1.

In Fig. 6D the illuminating light 10 is coupled into a near field probe. At the end of this probe an evanescent electromagnetic field is generated. Only one single particle 1 decouples propagating electromagnetic fields 11 from this evanescent wave, which are

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characterized by the spectral signature of the particle 1 and can be detected by a detector.

Another technique for detecting scattered light 11 from a single nanoparticle employs a flow through cell 14 with a cylindrical flow through channel as schematically shown in Fig. 7. On the inner surface 3 of this flow through channel an observed nanoparticle 1 is immobilised. This particle is coated on its surface with antibodies 2 which are specific for analyte molecules 4. When flowing a solution containing analyte molecules 4 through this flow through cell 14, analyte molecules 4 will bind to the antibodies 2 thereby modifying the particle plasmon resonance frequency of the particle 1. By using a flow through cell it is therefore possible to fish for single analyte molecules in a very small or very large volume (analyte harvesting).

Fig. 8 shows a further schematic example of a flow through cell 14 containing a flow through channel 16 with an inlet 17a and an outlet 17b. In or on the inner surface of substrate or section 3 arranged in the flow through channel 16 particles 1 are immobilized.

Particles 1 are deposited and arranged such, that they get into contact with a solution which is flown through the flow through channel 16. Of course, in the flow through channel 16, there may be arranged more than one section 3.

The flow through channel 16 is placed inside of a flow through cell 14 which at the same time constitutes a wave guide 15 for the incident interrogating light 10. With such an arrangement the particles are excited by the evanescent field of the incident light 10, which passes the refracting barrier between the

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wave guide 15 and the substrate 3. As only the eva- nescent field of the illuminating light 10 extends from the waveguide and excites the particles 1 the scattered light 11 is easily separated from the inci- dent light 10.

Scattered light 11 originating from particles 1 is then detected by an objective 8 of a microscope thereby enabling observation of a single individual particle.

Fig. 9 shows a picture of a further arrangement of a flow through cell with two crossing flow through channels 16 with dimensions between 10 um and 100um.

The height of the flow trough channels is 10 um thus allowing the measurement of 1 or few nanoliters of test liquid.

Fig. 10 demonstrates two different principles for multi-species detection (multiplexing). In Fig. 10A two different classes of gold particles 1,1'of dif- ferent sizes are shown. On the surface of particles 1,1'of different size, different antibodies 2,2' specific for different analyte molecules areimmobi- lized. Spectra IIa and IIb in Fig. 10A correspond to the scattering light spectra form particle 1 with or without bound analyte molecules, respectively. Spec- tra IIa'and IIb'in Fig. 10A correspond to the scat- tering light spectra from particle1'with or without bound analyte molecules, respectively. The spectra shown in Fig. 10A demonstrate, that by choosing dif- ferent particle sizes the resonance spectra are suf- ficiently separated to observe the resonance shift resulting from binding events for each of the parti- cles separately. Thus several analyte molecules can be detected simultaneously.

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In Fig. 10B a substrate 3 is partitioned into different zones a, b, c, d. In each of these zones parti-cles... are immobilized on the substrate 3 with different antibodies 2,2',2",2, respectively, coated on the surface of the particles. By spacially separating particles 1, 1',1"and 1'" different analytes may be sensed, even if the spectral characteristics of particles 1, 1', 1"andI'"are same or similar. However, particles... may also be different in their properties and each constitutes a separate class of particle with different spectral characteristics. Thus, several analytes can be detected simultaneously by spacial and optional spectral separation of the detection signals.

Fig. 11 shows schematically a method to enhance the signal, i. e. to enhance the shift of the plasmon resonance associated with a binding event to the binding entities (or binding sites) immobilized on the surface of the nanoparticles. In this figure particle 19 is added to the sample. Particle 19 can consist of gold or other materials or compositions and has immobilised to its surface a further antibody 18.

Particle 19 can also be a macromolecule, e. g. protein. The antibody 18 is specific to the analyte molecules 4 as are the antibodies 2 which are immobilised on the surface of the scattered light detectable particle 1. When an analyte molecule 4 binds to the antibody 2 it also may bind a gold particle 19 thereby bringing the gold particle 19 in close vicinity to particle 1. The gold particle 19 thus associated with particle 1 strongly influences the refractive index in close vicinity of the particle 1 and thus causes an increased shift of the plasmon reso-

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nance spectrum.

The present invention further comprises all kinds of competitive assays. Such competitive assays can be performed in two main formats which will be explained schematically with reference to figures 12 and 13.

In a first competitive format (fig. 12), an antibody 2 is immobilised on a plasmon resonant particle 1.

The analyte 4 contained in the sample competes with species 21 for binding to the immobilised antibody 2.

Species 21 can be the same molecular species as the analyte or a derivate, fragment, analogue or mimic of the analyte, capable of binding to the antibody 2.

Especially in cases where species 21 is of low molecular weight, it can be bound to a carrier 20 which can be a macromolecule, e. g. protein, polysaccharide, nucleic acid, synthetic polymer or a particle (metallic or other). Analyte 4 and species 21 coupled to carrier 20 exhibit a different influence on the particle plasmon resonance of particle 1 when bound to particle 1. Thus it is possible to discriminate between analyte 4 and species 21.

Fig. 12A shows a particle 1 with species 21 bound to the antibodies on its surface. The insert of fig. 12A depicts the scattered light spectrum IIb as generated by such a particle 1. The more analyte 4 is present in the sample, the less species 21 can bind to the antibody 1. This is shown in fig. 12B, where analyte molecules 4 have replaced species 21. Thereby the plasmon resonance is shifted to higher energy, i. e. from curve IIb to curve IIa as shown in the insert of fig. 12B.

To those skilled in the art, it is obvious that spe-

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cies 21 can be added to the particle 1 either before (as depicted in fig. 12), simultaneously with or after the addition of the sample. Also, the binding entity 2 is not limited to an antibody but may comprise any partner of a specific binding pair.

In a second competitive format (fig. 13), species 21 are immobilized directly or indirectly, e. g. via streptavidin/biotin, to a plasmon resonant particle 1. The bound species 21 competes with the analyte 4 from the sample for binding to free antibodies 2. Antibodies 2 are added and bound to the particles 1 prior to the addition of the sample containing analyte 4. Particle 1 then generates a particle plasmon spectrum lib as shown in the insert figure of fig.

13A. When adding the analyte 4, antibodies 2 are displaced from particle 1, thus effecting a shift of the particle plasmon resonance of particle 1 from curve IIb to curve IIa (see fig. 13B). The more analyte is present in the sample, the less antibody 2 remains bound to the species 21 resulting in a shift of the plasmon resonance to higher energy. The free antibody 2 can be added to the particle 1 either before, simultaneously with or after the addition of the sample. Also, the binding entity 2 is not limited to an antibody, but may comprise any partner of a specific binding pair.

Fig. 14 shows photographs and three-dimensional graphs of the scattering intensity of plasmon resonant particles on a substrate. In fig. 14A the picture was acquired using a wave guide measuring technique as described before (figs. 7-9), whereas in fig. 14B a dark field microscope as described before was used. In both cases the scattered light detectable particles were gold particles with a diameter of

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80 nm in undiluted blood serum.

Fig. 15 demonstrates the effect of particle shape on the characteristics of the plasmon resonance spectrum. Spectrum 11 is the plasmon spectrum generated by sphere-like nanoparticles. A rod-like particle exhibits a resonance spectrum11'. The spectrum of a rod-like particle is narrow with a sharp peak and therefore especially suitable for measuring small spectral shifts, whereas the spectrum of a spherelike particle is broader than the spectrum11'of the rod-like particle.

As the detection of a small shift is easier for narrow spectral resonances, selection of a suitable shape, e. g. a rod-like shape, of the nanoparticles greatly improves measurement of plasmon resonance shifts.

Fig. 16 shows in fig. 16A two particles 1,1' which are spaced by a distance d from each other. Such two particles interact with each other, particularly if distance d is less than the particle diameter, preferably less than the particle radius.

If the particles interact strongly enough, collective electron oscillations in both particles can be excited resulting in longitudinal (with respect to the connecting axis) or transversal oscillations. These oscillations can be separated by polarizers and observed. The particle plasmon spectrum observed from such a particle stucture is shown in fig. 16A.

Fig. 16B displays the spectra of a single particle 1 in air (n = 1) as curve 30, whereas curve 31 is the particle plasmon resonance spectrum of such a single

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particle in water(H_2O, n = 1,33). The change in refractive index from air to water leads to a shift of the spectrum peak of 54 meV.

If the longitudinal mode of a pair of coupled particles as shown in fig. 16A with particle diameters of about 40 nm and a distance d between the particles of less than 20 nm is measured, the transfer of such particle structure from air (n = 1, see curve 32 in fig. 16C) to water (n = 1,33, see curve 33 in fig.

16C). results in a shift of 74 meV, i. e. particle structures comprising several coupled particles show a larger shift than single particles.

Thus, it is shown, that particle structures comprising several coupled particles act physically as one scattering entity. Together they exhibit unique spectral characteristics suitable for use in the present

invention as measure for changes in the vicinity of the particle structure.

Such particle structures can be manufactured by arranging particles in defined, specific distances to each other or by generation of particle structures with specific features on a substrate, e. g. by lithography.

In the following various examples of inventive assays and experimental results will be shown.

Example 1 Fig. 17 demonstrates binding of BSA-digoxigenin (BSA = bovine serum albumin) to gold particles with a diameter of 40 mm. These gold particles were coated

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with about 50 antibodies M19-11 per particle against digoxigenin.

First, plasmon resonance was measured with a microscope with a water immersion objective lense in a solution containing bovine serum albumin (BSA) at a concentration of 0.2 mg/ml. The observed spectrum is shown as solid line in fig. 17B.

Further, 10 pi BSA-digoxigenin was added to a solution containing the above mentioned functionalized gold particles to a final concentration of RPLAdigoxigenin of 3,0x10-6mol/1. The resulting spectrum after 71 min is shown as dashed line in Fig. 17B.

Fig. 17A displays the time course of the development of the plasmon resonance shift after addition of BSAdigoxigenin at timet=O. Within 40 min a shift of the plasmon resonance peak of 10 meV to longer wavelength occurred.

The observed shift can not be explained by changes in measuring conditions during the incubation with RPLAdigoxigenin. Such effects amount to less than +/-1 meV. The shift is therefore largly due to binding of BSA-digoxigenin to the gold particles.

Example 2 Figs. 18 and 19 show results of a binding study of streptavidin to single gold nanoparticles funtionalized with biotin.

Measurements were done with a dark field microscope setup with water immersion objective lense(100K,

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NA=1.0). Scattered light from single individual functionalized gold nanoparticles of 40 nm diameter, electrostatically attached on a cover slip were observed. White light from a 100 W halogen lamp was focused under large angles onto the sample using a dark field condenser with high numerical aperture (NA=1.2- 1.4). The scattered light of a single nanoparticle in the focus was collected, spectrally resolved in a grating spectrometer and detected with a nitrogen cooled and back illuminated CCD camera.

For preparation of the, functionalized gold particles, first nanoparticles with a diameter of 40 nm were functionalized with biotinylated bovine serum albumin (biotin-BSA) molecules (MW : 67000 D). As analyte to be detected streptavidin (MW: 52000 D), a tetrameric protein which can bind up to four biotin molecules was used. The biotin-BSA coated gold nanoparticles were immobilized onto the surface of a silanized glass substrate and covered by 10 mM Tris [NH2C (CH20H)3]/BSA buffer solution (pH 8.0, 0.5 mg/ml BSA). Silanization together with BSA in the buffer solution intended to prevent non-specific streptavidin binding on the glass substrate and the objective lens. For an individual functionalized nanoparticle a scattering spectrum at a resonance position of 2.282 eV was found as shown in fig. 18A and fig.

18B as solid curve.

Then, 10pi potassium phosphate buffer (pH 6.5, 20 mM) containing6x10-5mol/1 streptavidin was added to a final streptavidin concentration of2x10-6mol/1. The assay was then incubated for 30 min. This resulted in a 5 meV spectral shift of the nanoparticle plasmon resonance curve as shown by the dashed curve in fig. 18B and, on an extended scale,

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in fig. 18A. This shift is also clearly seen in fig.

18C which shows the differential spectrum between the solid curve and the dashed curve in fig. 18B. The shape of this differential scattering spectrum in fig. 18C indicates that the addition of streptavidin included

a pure shift of the nanoparticle plasmon resonance. The experimentally determined shift of 5 meV agrees well with the theoretically expected value of 7.5 meV for free nanoparticles. The difference can be easily explained by the fact that in the experiment the nanoparticles are not free, but attached to a substrate. Accordingly one third of the functionalized nanoparticle surface is not available for the analyte molecules.

To ensure that the observed redshift is not caused by the added potassium phosphate storage buffer of the streptavidin, supplementary control experiments were performed by monitoring the resonance position of an individual nanoparticle in Tris/BSA buffer for 15 min (see fig. 19, squares). At t=O min 10 ul of potassium phosphate buffer (equal to the streptavidin storage buffer) was added to the solution. Subsequent monitoring of the resonance position for 45 min did not show any resonance shift and hence buffer induced changes of the nano-environment can be ruled out. It is thus concluded that the observed redshift is a direct consequence of streptavidin binding.

Further the kinetics of the specific binding processes were measured as shown in fig. 19 (circles and triangles). At time t=O min, 3.12 mg/ml streptavidin (corresponding to a molar concentration of6x10-5mol/1) was added to a Tris/BSA buffer solution (10 mM, pH 8.0, 0.5 mg/ml), resulting in a final streptavidin concentration of 100 ug/ml(2x10-6 mol/1). One

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minute after streptavidin addition, the NPP resonance position exhibited a significant redshift (fig. 19, circles). It saturated with increasing incubation time and reached a constant total displacement of 5meV after approximately 15 min. Data points plotted as triangles in Fig. 19 correspond to a lower concentration, where streptavidin was added at t=O min to a final concentration of 50 ug/ml(lx10-6mol/1). Compared to the higher concentration (fig. 19, circles), the redshift evolved on a slower time scale and the total resulting nanoparticle plasmon shift was reduced.

In the following it is shown that the observed time behaviour is governed by the kinetics of the binding reaction and is not limited by diffusion. The rate AN/At of streptavidin molecules impinging onto a free nanoparticle due to diffusion is given byAN/At=4nDrC, where D=7.4x10-7cm2/s is the diffusion constant of streptavidin, r=23 nm the radius of a functionalized nanoparticle andC=lx10-6 mol/1 the molar concentration of free streptavidin molecules.

For diffusion limited kinetics this rate would lead to a completely filled streptavidin shell around the functionalized nanoparticle in less than one second.

Consequently the observed time evolution of the nanoparticle plasmon resonance shift is not determined by diffusion, but by the kinetics of the binding reaction. For our dataset we applied a first order model for binding analyte molecules to acceptor sites. The deduced affinity constant of Ka=106 1/mol is much lower than expected for free biotinstreptavidin binding. We attribute this low value to the fact that the biotin molecules are located within a disordered protein network of BSA molecules. This leads to limited accessibility of biotin molecules

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and thus lower association rates. In addition, the BSA matrix may cause enhanced dissociation of bound biotin-streptavidin complexes. Low association rates and enhanced dissociation rates due to surface effects lead to drastically reduced affinity constants.

In conclusion the examples shown in fig. 18 and fig.

19 demonstrate a biotin-streptavidin affinity biosensor using light scattering spectroscopy of single gold nanoparticles.

METHODS, DEVICE AND INSTRUMENT FOR DETECTION OF ANALYTES

Claims of **WO2004042403**

Claims 1. A method for detection of an analyte in a sam- ple, comprising the steps of: providing at least one particle structure de- tectable by light scattering and coated with binding entities for said analyte; contacting the at least one particle structure with the sample, illuminating the at least one particle structure and measuring the scattered light emitted by in- dividual particle structures at least twice and determining a change in the spectral signature of the scattered light as a measure of the pres- ence and/or quantity of the analyte in the sam- ple.

2. A method for detection of an analyte in a sam- ple, comprising the steps of: providing at least one particle structure de- tectable by light scattering and coated with substances competing with said analyte for bind- ing to specific binding entities; contacting the at least one particle structure with the sample and said binding entities, illuminating the at least one particle structure and measuring the scattered light emitted by in- dividual particle structures at least twice and determining a change in the spectral signature of the scattered light as a measure of the pres- ence and/or quantity of the analyte in the sam- ple.

3. A method for detection of two or more different analytes in a sample, comprising the steps of:

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providing at least one particle structure of each of two or more classes of particle struc- tures detectable by light scattering, the particle structures of each class of parti- cle structures being coated with specific bind- ing entities for one of these analytes; contacting the particle structures with the sam- ple, illuminating the particle structures, and meas- uring the scattered light emitted by individual particle structures at least twice and determining a change in the spectral signature of the scattered light as a measure of the pres- ence and/or quantity of the analytes in the sam- ple.

4. A method for detection of two or more different analytes in a sample, comprising the steps of: providing at least one particle structure of each of two or more classes of particle struc- tures detectable by light scattering, the particle structures of each class of parti- cle structures being coated with substances com- peting with one of said analytes for binding to specific binding entities; contacting the particle structures with the sam- ple and said binding entities, illuminating the particle structures and measur- ing the scattered light emitted by individual particle structures at least twice and determining a change in the spectral signature of the scattered light as a measure of the pres- ence and/or quantity of the analyte in the sam- ple.

5. The method according to claim 1 or 3, wherein a competitive agent competing with the analyte for

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binding to the binding entities is contacted with the particle structures before, duringand/or after contacting the particle structures with the sample.

6. The method according to claim 2 or 4, wherein said binding entities are contacted with the particle structures before, during or after con- tacting the particle structures with the sample.

7. The method according to claim 3 or 4, wherein the particle structures of different classes have a different size, shape, structure, mate- rial and/or composition.

8. The method according to one of the preceding claims, wherein after contacting the particle structures with the sample, the particle struc- tures are further contacted with a signal en- hancing agent specifically binding to the ana- lyte or analytes and modifying the scattering characteristics of the particle structure when bound to the particle.

9. The method according to the preceding claim, wherein the signal enhancing agent comprises a metal, alloy, semiconductor, glass, latex parti- cle or organic substance, e. g. proteins, poly- saccharides, nucleic acids, organic synthetic or natural polymers as label.

10. The method according to one of the preceding claims, wherein the binding entities and/or sig- nal enhancing agents comprise an antibody, anti- gen binding fragment-of an antibody, peptide, protein, antigen, nucleic acids, peptide nucleic acids, or artificial binding agent.

ously or at one or several distinct time points.

27. The method according to one of the preceding claims, wherein the difference spectrum between or the ratio of the scattering spectra taken at different time points are calculated in order to determine a shift of wavelength in the scattered light spectrum.

28. The method according to one of the preceding claims, wherein particle structures with a di- ameter or an average diameter of 1 to 500 nm, preferably between 5 and 300 nm are used.

29. The method according to the preceding claim, wherein particle structures with a diameter or an average diameter of 10 nm to 150 nm are used.

30. The method according to one of the preceding claims, wherein particle structures made of or comprising metals, noble metals, alloys, semi- conductors or a heterostructure thereof are used.

31. The method according to one of the preceding claims, wherein particle structures with a me-

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tallic, organic polymer, latex, glass core or metallic, organic polymer, latex or glass sur- face layer are used.

32. The method according to one of the preceding claims, wherein particle structures of rod-, el- lipsoid-or sphere-like shape are used.

33. A device for the detection of an analyte in a sample, comprising at least one particle structure detectable by light scattering and coated with binding enti- ties for said analyte, the at least one particle structure being arranged such, that scattered light emitted by individual particle structures can be detected.

34. A device for detection of an analyte in a sam- ple, comprising : at least one particle structure detectable by light scattering and coated with substances com- peting with said analyte for binding to specific binding entities ; the particle structures being arranged such, that scattered light emitted by individual par- ticle structures can be detected.

35. A device for detection of two or more different analytes in a sample, comprising at least one particle structure for each of two or more classes of particle structures detect- able by light scattering, the particle struc- tures of each class of particle structures being coated with binding entities for one of these analytes, the particle structures being arranged such, that scattered light emitted by individual par-

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ticle structures can be detected.

36. A device for detection of two or more different analytes in a sample, comprising: at least one particle structure of each of two or more classes of particle structures detect- able by light scattering, the particle structures of each class of parti- cle structures being coated with substances com- peting with one of said analytes for binding to specific binding entities; the particle structures being arranged such, that scattered light emitted by individual par- ticle structures can be detected.

37. A device according to one of claims 33 to36,. wherein the particle structures are deposited on a substrate.

38. The device according to one of claims 35 or 36, wherein the particle structures of different classes have a different size, shape, structure, materialand/or compostion.

39. The device according to one of claims 33 to 38, wherein the at least one particle structure is deposited with a distance from other particle structures of at least the spacial resolution of the optical detection means of the scattered light.

40. The device according to one of claims 33 to 39, wherein the particle structures are arranged with a distance of between 1 and 500 um between each other.

41. The device according to one of claims 33 to 40, wherein the particle structures have a diameter

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ticle structures can be detected.

36. A device for detection of two or more different analytes in a sample, comprising: at least one particle structure of each of two or more classes of particle structures detect- able by light scattering, the particle structures of each class of parti- cle structures being coated with substances com- peting with one of said analytes for binding to specific binding entities; the particle structures being arranged such, that scattered light emitted by individual par- ticle structures can be detected.

37. A device according to one of claims 33 to36,. wherein the particle structures are deposited on a substrate.

38. The device according to one of claims 35 or 36, wherein the particle structures of different classes have a different size, shape, structure, material and/or compostion.

39. The device according to one of claims 33 to 38, wherein the at least one particle structure is deposited with a distance from other particle structures of at least the spacial resolution of the optical detection means of the scattered light.

40. The device according to one of claims 33 to 39, wherein the particle structures are arranged with a distance of between 1 and 500 um between each other.

41. The device according to one of claims 33 to 40, wherein the particle structures have a diameter

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or an average diameter of 1 to 500nm, prefera- bly between 5 nm to 300 nm, preferably between 10 nm to 150 nm.

42. The device according to one of claims 33 to 41, wherein the particle structures are made of or comprise metals, noble metals, alloys, semicon- ductors or heterostructures thereof.

43. The device according to one of claims 33 to 42, wherein the particle structures have a metallic, organic, polymer, latex or glass core or metal- lic, organic, polymer, latex or glass surface layer.

44. The device according to one of claims 33 to 43, wherein the particle structures have a rod-like, ellipsoid-like or sphere-like shape.

45. The device according to one of claims 33 to 44, wherein the binding entities comprise one of the group of antibody, antigen binding fragment of an antibody, peptide, protein, antigen, nucleic acids, peptide nucleic acids or artificial bind- ing agent.

46. The device according to one of claims 33 to 45, further comprising a flow-through cell.

47. The device according to claim 46, wherein the substrate is arranged on or is constituting a part of the lumenal wall of a flow-through cell, the particle structures being exposed to the lu- men of the flow-through cell.

48. The device according to claim 37,46 or 47, wherein the substrate comprises a waveguide for guiding light from a means for illuminating the

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particle structure to the particle structure.

49. The device according to the preceding claim, wherein the waveguide is arranged such, that an evanescent electromagnetic field of the illumi- nating electromagnetic wave extends to the par- ticle structure.

50. The device according to claim 46 or 47, wherein the lumen of the flow-through cell is a wave guide.

51. A measuring instrument comprising a device ac- cording to one of claims 33 to 50, further com- prising means for illuminating the particle structures with monochromatic or polychromatic light and/or means for detecting scattered light emitted by individual particle structures.

52. The instrument according to the preceding claim, wherein said means for illuminating the particle structures comprises one or more incandescent lamps, fluorescent lamps, light emitting diodes, semiconductor lasers and/or lasers.

53. The instrument according to claim 51 or 52, wherein the particle structures are illuminated using dark field illumination, illumination in reflection, total internal reflection, near field illumination and/or a wave guide.

54. The instrument according to one of claims 51 to 53, wherein the substrate is arranged in a dark field microscope, a reflection microscope, a re- flection microscope with crossed polarizers, to- tal internal reflection microscope and/or scan- ning near field optical microscope (SNOM).

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55. The instrument according to one of claims 51 to 54, wherein the means for detecting scattered light comprises one or several photodiodes, a diode array, one or several photomultipliers and/or a CCD-camera.

56. The instrument according to one of claims 51 to 55, further comprising means for spectral reso- lution of the scattered light.

57. Use of a method, a device or an instrument ac- cording to one of the preceding claims in analy- sis, medicine, clinical diagnostics, veterinary diagnostics, pharmacology, environmental analy- sis and/or biochemistry.

58. Use of a method, a device or an instrument ac- cording to one of claims 1 to 56 for screening for analytes or search for therapeutic agents or others.

59. Use of a method, a device or an instrument ac- cording to one of claims 1 to 56 for the selec- tive detection of haptens, peptides, proteins, antigens, antibodies or fragments thereof, polynucleotides, oligonucleotides, DNA, RNA.

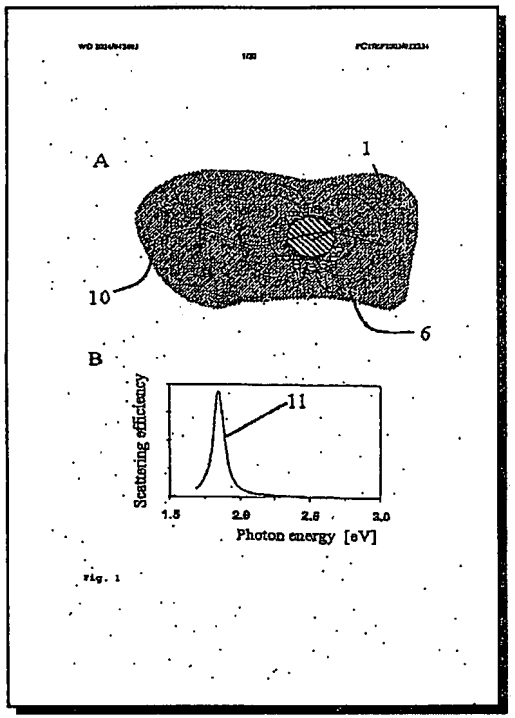

Fig. 1

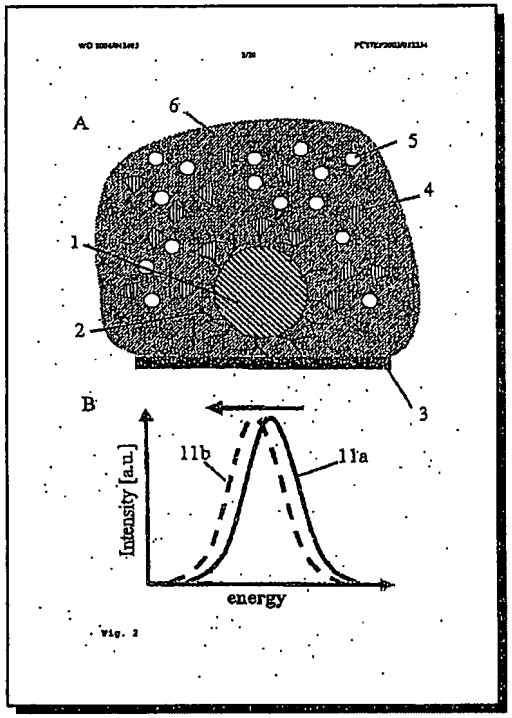

Fig. 2

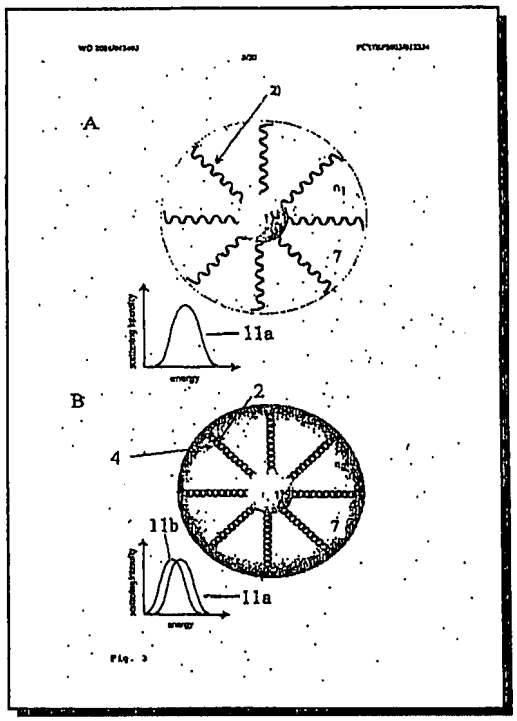

Fig. 3

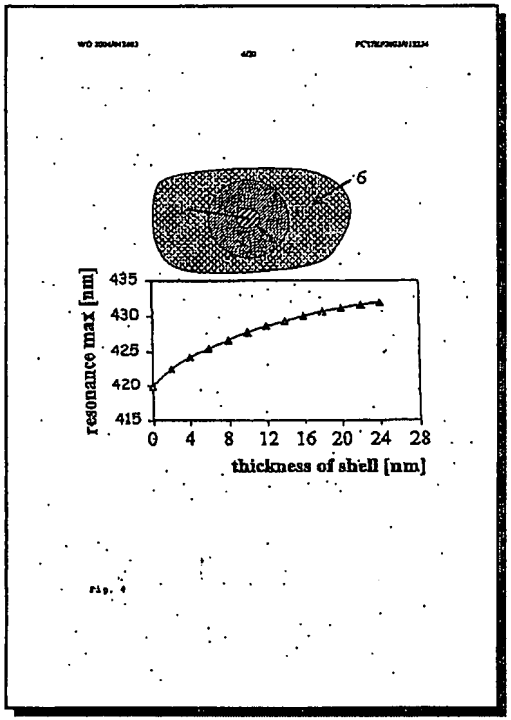

Fig. 4

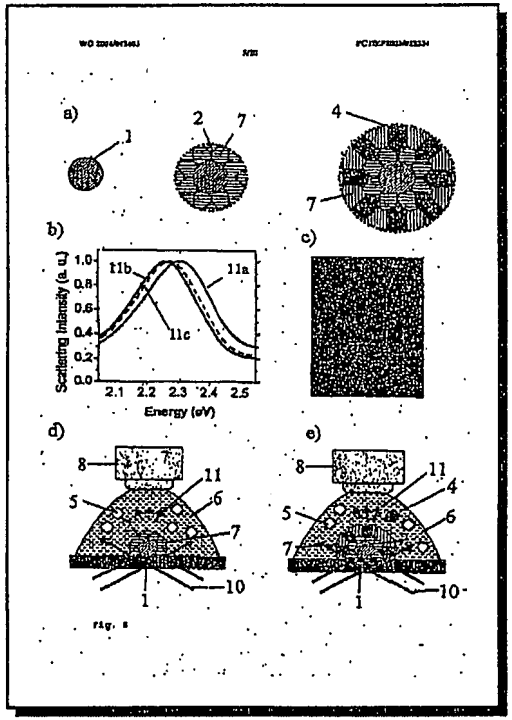

Fig. 5

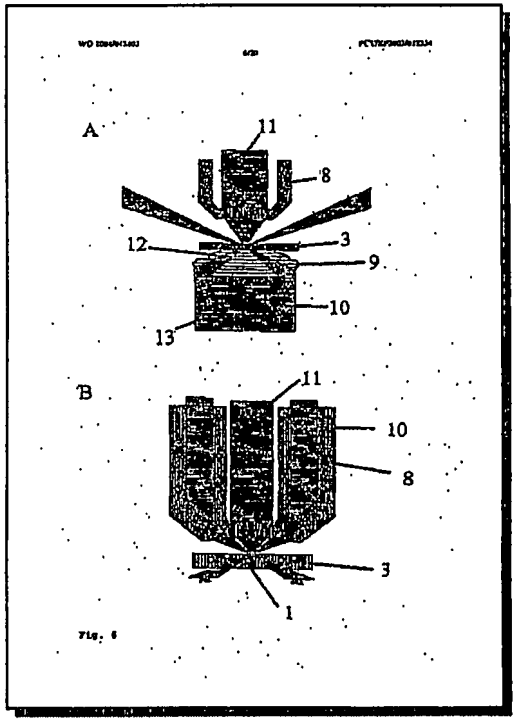

Fig. 6

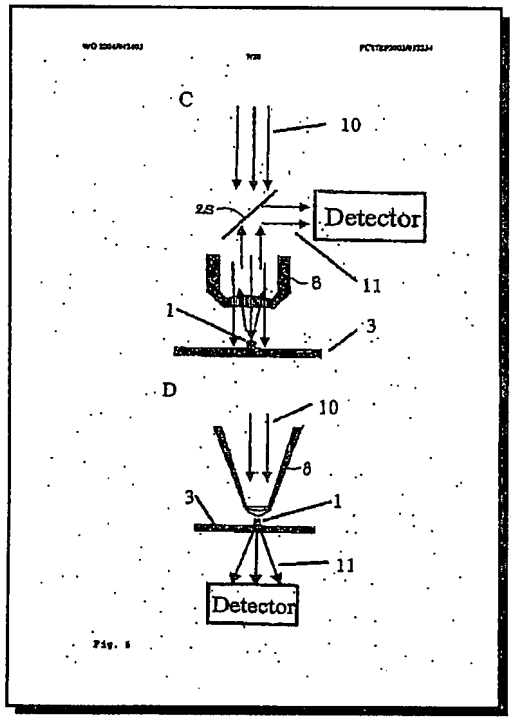

Fig. 6

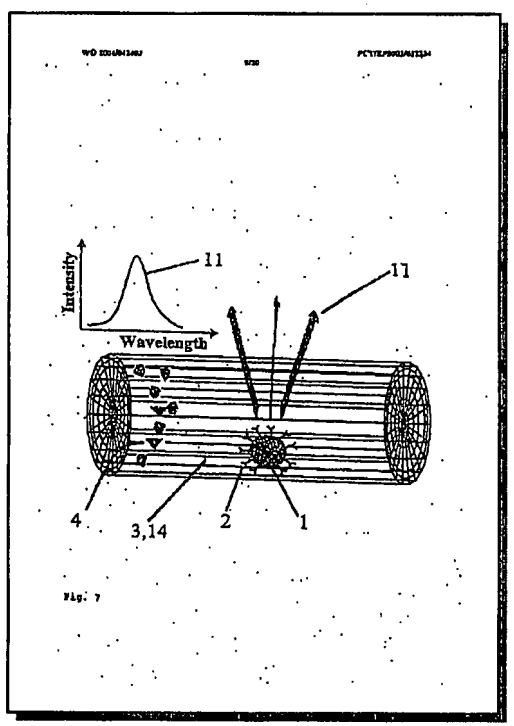

Fig. 7

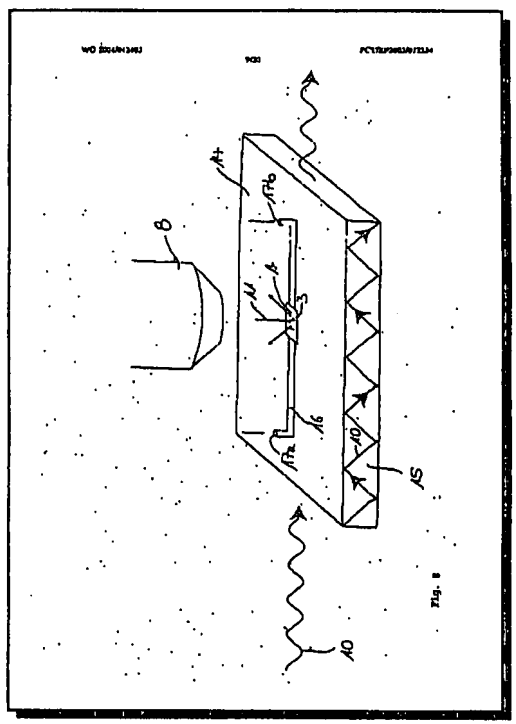

Fig. 8

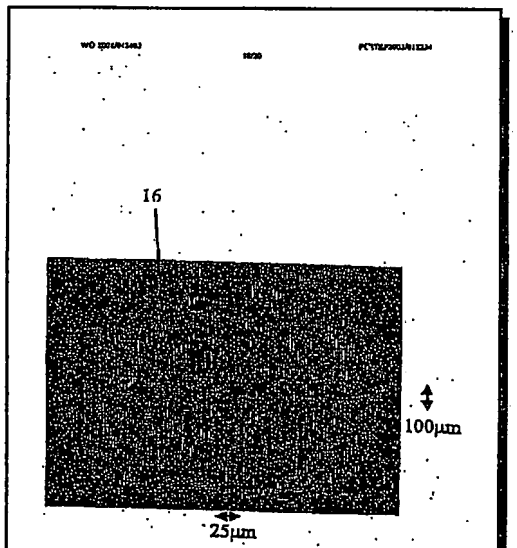

Fig. 9

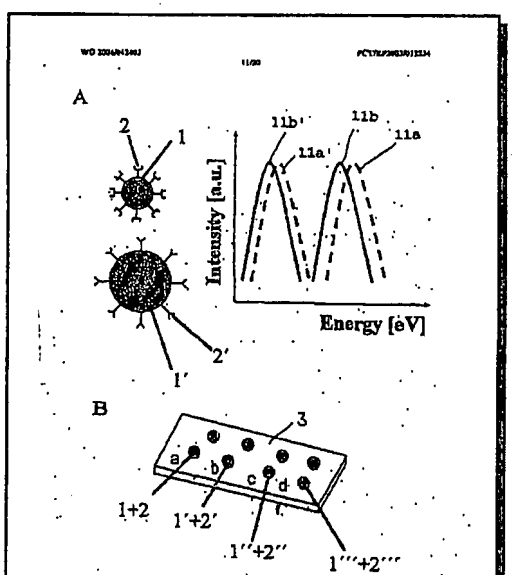

Fig. 10

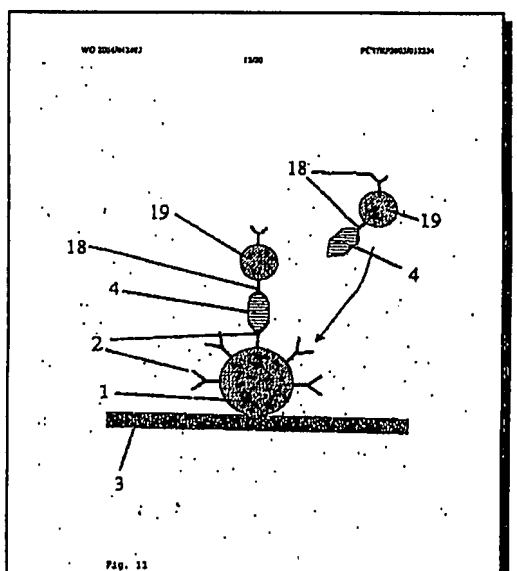

Fig. 11

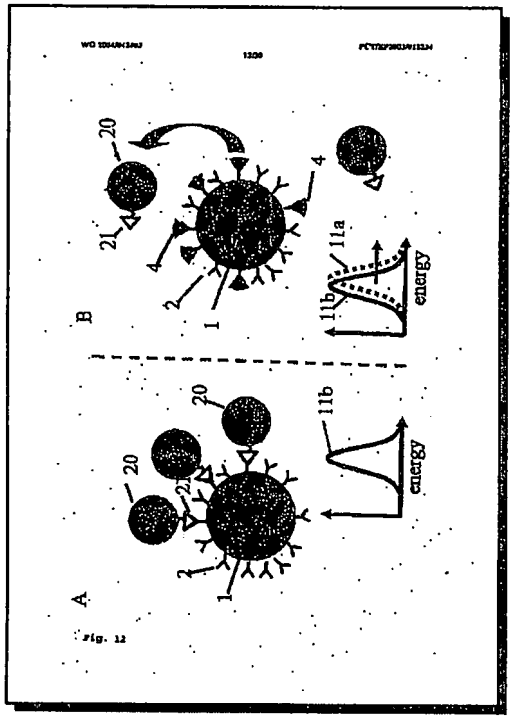

Fig. 12

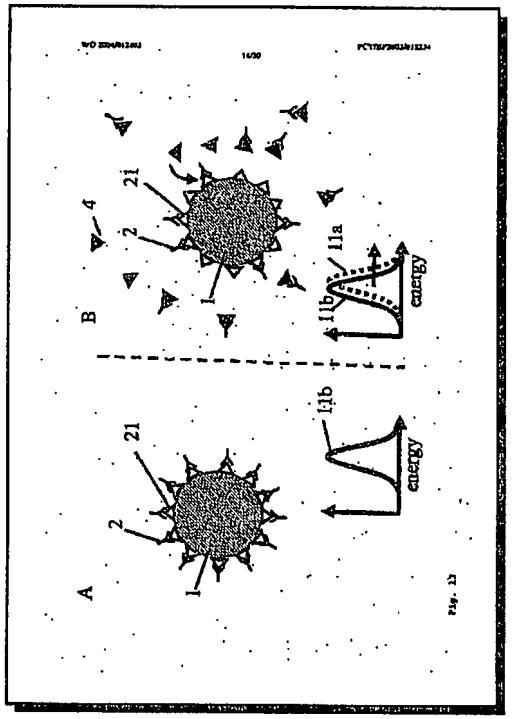

Fig. 13

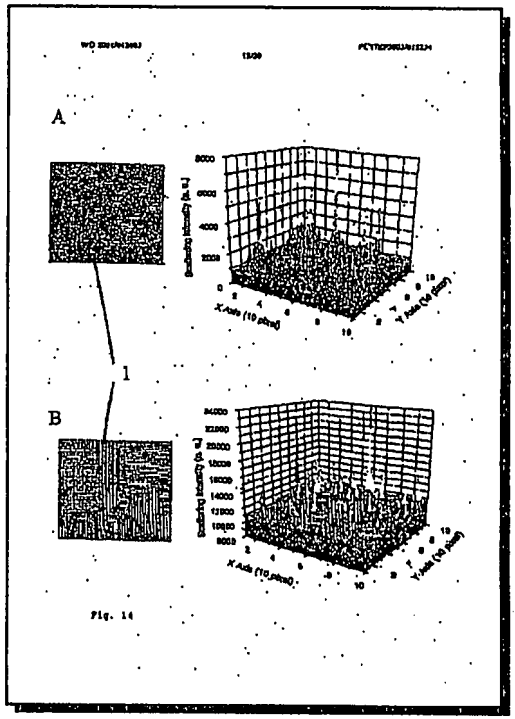

Fig. 14

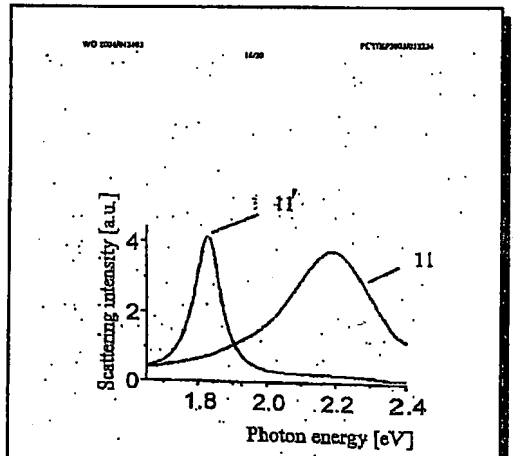

Fig. 15

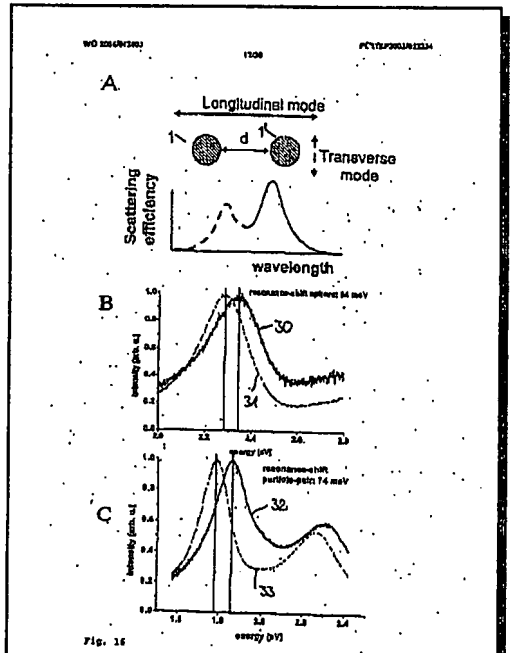

Fig. 16

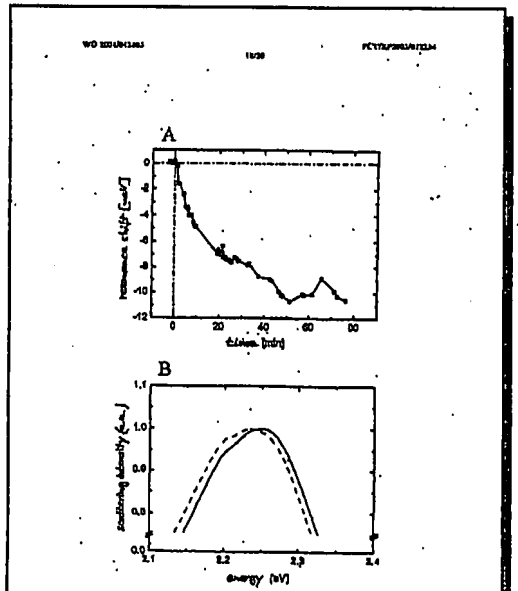

Fig. 17

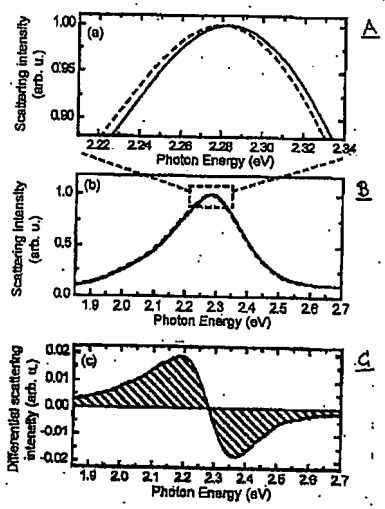
(a)
A
Scattering intensity (arb. u.)
1.00
0.95
0.90
2.22 2.24 2.26 2.28 2.30 2.32 2.34
Photon Energy (eV)
(b)
B
Scattering intensity (arb. u.)
1.0
0.5
0.0
1.9 2.0 2.1 2.2 2.3 2.4 2.5 2.6 2.7
Photon Energy (eV)
(c)
C
Differential scattering intensity (arb. u.)
0.02
0.01
0.00
-0.01
-0.02
1.9 2.0 2.1 2.2 2.3 2.4 2.5 2.6 2.7
Photon Energy (eV)

Fig. 18

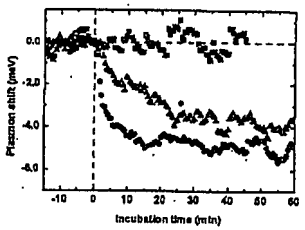
Plasmon shift (meV)
0.0
-2.0
-4.0
-6.0
-10 0 10 20 30 40 50 60
Incubation time (min)

Fig. 19